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Motor function recovery: deciphering a regenerative niche at the neuromuscular synapse

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ABSTRACT

The coordinated movement of many organisms relies on efficient nerve–muscle communication at the neuromuscular junction (NMJ), a peripheral synapse composed of a presynaptic motor axon terminal, a postsynaptic muscle specialization, and non-myelinating terminal Schwann cells. NMJ dysfunctions are caused by traumatic spinal cord or peripheral nerve injuries as well as by severe motor pathologies. Compared to the central nervous system, the peripheral nervous system displays remarkable regenerating abilities; however, this capacity is limited by the denervation time frame and depends on the establishment of permissive regenerative niches. At the injury site, detailed information is available regarding the cells, molecules, and mechanisms involved in nerve regeneration and repair. However, a regenerative niche at the final functional step of peripheral motor innervation, i.e. at the mature neuromuscular synapse, has not been deciphered. In this review, we integrate classic and recent evidence describing the cells and molecules that could orchestrate a dynamic ecosystem to accomplish successful NMJ regeneration. We propose that such a regenerative niche must ensure at least two fundamental steps for successful NMJ regeneration: the proper arrival of incoming regenerating axons to denervated postsynaptic muscle domains, and the resilience of those postsynaptic domains, in morphological and functional terms. We here describe and combine the main cellular and molecular responses involved in each of these steps as potential targets to help successful NMJ regeneration.

Key words: neuromuscular junction, synapse, denervation, regeneration, niche, motor neuron, muscle fibre, contraction

CONTENTS

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I. INTRODUCTION

The establishment of functional contacts between motor neurons and skeletal muscles at the neuromuscular junction (NMJ) is essential for the coordinated movement of a variety of organisms. The vertebrate NMJ is possibly the best-characterized synapse due to its relative simplicity and easy experimental accessibility (Sanes & Lichtman, 2001). The NMJ is composed of three closely associated cellular components: a presynaptic motor nerve terminal, a postsynaptic muscle specialization, and non-myelinating terminal Schwann cells. The experimental advantages of the NMJ have facilitated the identification of the molecules and mechanisms that regulate synapse formation, growth, maturation, maintenance, and function. Indeed, synaptic ultrastructure and the principles of synaptic transmission were first characterized using the frog NMJ (Birks, Huxley & Katz, 1960; Katz & Miledi, 1969; Katz, 1971). Importantly, failures in NMJ pre- or postsynaptic components, as well as traumatic spinal cord and nerve injuries, result in motor-associated pathologies. Along with undermining the quality of life of patients, conditions resulting in neuromuscular failure represent major costs for health systems. The peripheral nervous system has much higher regenerative capabilities than the central nervous system and important research efforts have concentrated on describing and repairing peripheral nerves at the injury site. This focus has been based on experimental evidence obtained from animal models showing that, once repaired, motor axons reach skeletal muscles and motor activity is recovered. However, this is not the case in human patients, where natural differences in body size often imply a considerable delay before muscles can be potentially reinnervated. Supporting this notion, animal paradigms of delayed NMJ regeneration display a morphological synaptic repair that is not associated with positive functional motor outcomes, suggesting synaptic rather than regenerative failures after critical time points of regeneration (Ma *et al.*, 2011; Sakuma *et al.*, 2016). Therefore, there is a need to reshape this conceptual view in order to define a missing regenerative niche at the final functional step of peripheral motor innervation, i.e. at the mature neuromuscular synapse. With this aim, in the first section of this review we will describe the main cellular and molecular mechanisms driving NMJ assembly, maturation, and maintenance as a crucial initial step to elucidate their potential role in the regenerative process.

(1) Early NMJ synaptogenesis

The main morphological features leading to embryonic NMJ formation have been well described, particularly in murine species. As trunks of motor nerves penetrate peripheral regions where myotubes have been recently differentiated, motor axons branch to innervate a variable number of skeletal muscle fibres in an endplate band. Presynaptic differentiation is accompanied by morphological changes in motor terminals that contact the muscle fibre and begin to accumulate synaptic vesicles containing acetylcholine and other presynaptic components (a in Fig. 1A). Postsynaptic differentiation is characterized by: (*i*) increased expression of several postsynaptic proteins, including the nicotinic acetylcholine receptors (AChRs), specifically in the few myonuclei localized beneath the junction (named 'subsynaptic nuclei'), and (*ii*) the aggregation of these proteins (a hallmark of postsynaptic differentiation) in a small invaginated and folded fraction of the muscle membrane to shape the nascent NMJ (Marques, Conchello & Lichtman, 2000; Sanes & Lichtman, 2001). The assembly of functional vertebrate NMJs relies on signals and extracellular matrix molecules derived from both motor axons and muscle fibres. Initial studies suggested a neural control of early postsynaptic assembly by describing two motor neuron-derived proteins, neuregulins and agrin, as key signals for NMJ synaptogenesis. Neuregulins were described as transcriptional regulators of AChRs and other postsynaptic proteins through their binding to members of the ErbB family of receptors (named after the erythroblastic leukemia viral oncogene, with which these receptors are homologous) expressed in muscle fibres (Fischbach & Rosen, 1997; Trinidad, Fischbach & Cohen, 2000). However, the requirement of neuregulins for early NMJ assembly was later questioned, based on the presence of normal NMJs in animals where ErbB2 and ErbB4 were absent (Escher *et al.*, 2005). Agrin is a key regulator of AChR aggregation by binding a muscle protein complex containing the muscle-specific tyrosine kinase receptor MuSK (Valenzuela *et al.*, 1995; DeChiara *et al.*, 1996) and the co-receptor lipoprotein receptor related protein-4 (LRP4) (Kim *et al.*, 2008; Zhang *et al.*, 2008) (a in Fig. 2A). Activation of the agrin/LRP4/MuSK complex recruits crucial intracellular proteins for postsynaptic assembly (detailed in Burden, Huijbers & Remedio, 2018). These include the MuSK-binding proteins Dok7 (downstream of tyrosine kinase 7) and Tid1 (the mammalian homologue of the *Drosophila* tumorous imaginal disc 1 protein) (Okada *et al.*, 2006; Linnoila *et al.*, 2008), as well as the AChR-binding protein rapsyn (Phillips *et al.*, 1993). Mice deficient in MuSK, rapsyn, Dok7, or Tid1 show no signs of postsynaptic differentiation (Phillips *et al.*, 1993; DeChiara *et al.*, 1996; Okada *et al.*, 2006; Linnoila *et al.*, 2008), demonstrating the crucial role of the agrin/LRP4/MuSK pathway for neuromuscular synaptogenesis. Even though the diaphragm muscle of agrin-null animals shows impaired NMJ morphology (Gautam *et al.*, 1995), they display AChR aggregates in a central end-plate band during the earliest (aneural) stages of NMJ development (Lin *et al.*, 2001). Moreover, skeletal muscles of animal models lacking motor neurons also assemble a central 'pre-pattern' of AChR clusters (Lin *et al.*, 2001; Yang *et al.*, 2001; Pun *et al.*, 2002), a finding that initially suggested potential myogenic control of early AChR aggregation. In the zebrafish (*Danio rerio*) NMJ, it was later demonstrated that mesenchymal cells in regions where muscles are being differentiated secrete Wnt11r (wingless-related integration site 11r), which binds to and activates MuSK to induce the pre-patterning of

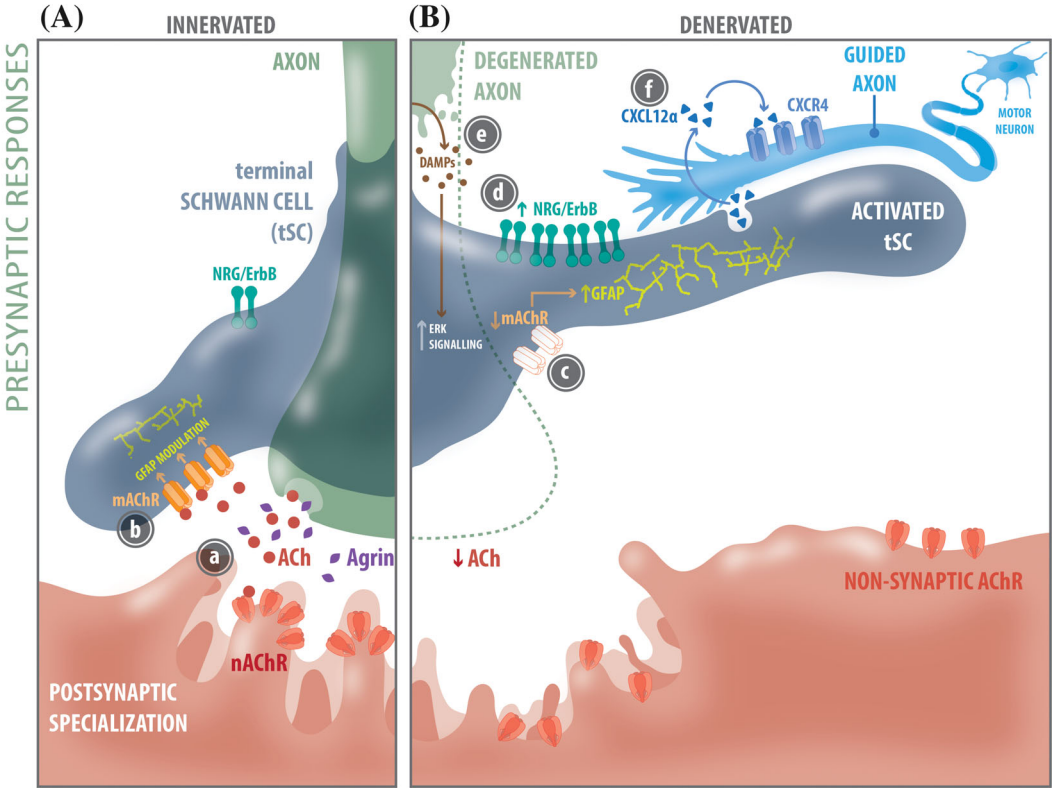

Fig 1. Crucial cells and molecules mediating presynaptic responses in the regenerative niche at the vertebrate neuromuscular junction (NMJ). In innervated mature NMJs (A), trophic factors such as Agrin and the neurotransmitter acetylcholine are released from the presynaptic nerve terminal. ACh diffuses at the synaptic cleft and binds to nAChRs at the postsynaptic specialization to trigger muscle fibre contraction (a). In addition, released ACh binds to and activates mAChRs in terminal Schwann cells, modulating GFAP expression (b). Upon NMJ denervation (B) the degenerated nerve terminal undergoes Wallerian degeneration, leading to the denervation of the skeletal muscle fibre. Remarkably, terminal Schwann cells become active and extend processes which guide regenerating axons to the denervated NMJ. Molecularly, as ACh is no longer present at the synapse, mAChRs become inactive (c), leading to GFAP up-regulation with the concomitant activation of terminal Schwann cells. Denervation also deprives the NMJ microenvironment of retrograde and anterograde signals that help NMJ stability (d, e). NRG/ErbB signalling is increased by up-regulation of ErbB receptors in terminal Schwann cells (d). DAMPs are released from the degenerated axon as injury responses (e). These molecules directly activate terminal Schwann cells *via* an ERK signalling-dependent mechanism. The chemokine CXCL12α is secreted by terminal Schwann cell processes, which signals through its receptor CXCR4 expressed in the regenerating axons (f). Thus, a chemotaxis-related migratory response is achieved on terminal Schwann cell bridges. ACh, acetylcholine; AChR, acetylcholine receptor; CXCL12α, C-X-C motif chemokine 12 alpha; CXCR4, C-X-C chemokine receptor type 4; DAMPs, damage-associated molecular patterns; ErbB, receptor homologous for erythroblastic leukemia viral oncogene; ERK, extracellular signal-regulated kinase; GFAP, glial fibrillary acidic protein; mAChR, muscarinic acetylcholine receptor; nAChR, nicotinic acetylcholine receptor; NRG, Neuregulin.

AChRs (Jing *et al.*, 2009) and initiates synaptogenesis through MuSK endocytosis (Gordon *et al.*, 2012). In the same model, it was shown that some pre-existing AChR clusters located in dorsal muscles (i.e. proximal to the spinal cord) are contacted and stabilized by motor axons for synapse formation, while AChR aggregates of more distal ventral muscles are assembled only after the motor axon contacts them (Flanagan-Steet *et al.*, 2005). Most pre-patterned AChR clusters are not contacted by nerve terminals and become dispersed by an axon-dependent disaggregation activity triggered by the neurotransmitter acetylcholine (Marques *et al.*, 2000; Lin *et al.*, 2005; Misgeld *et al.*, 2005; Wu, Xiong & Mei, 2010). Genetic approaches led to the conclusion that the main role of aneurally pre-patterned AChR clusters is likely to determine the muscle membrane domains where neuromuscular synapses will be assembled (Ponomareva *et al.*, 2006; Vock, Ponomareva & Rimer, 2008).

A third cellular party, Schwann cells, also participate in NMJ establishment. During embryonic and perinatal NMJ development, neural crest-derived myelinating Schwann cells migrate and wrap around branched motor axons that have recently innervated nascent myotubes. In turn, each motor nerve ending becomes capped by 3–6 non-myelinating 'terminal Schwann cells' from perinatal

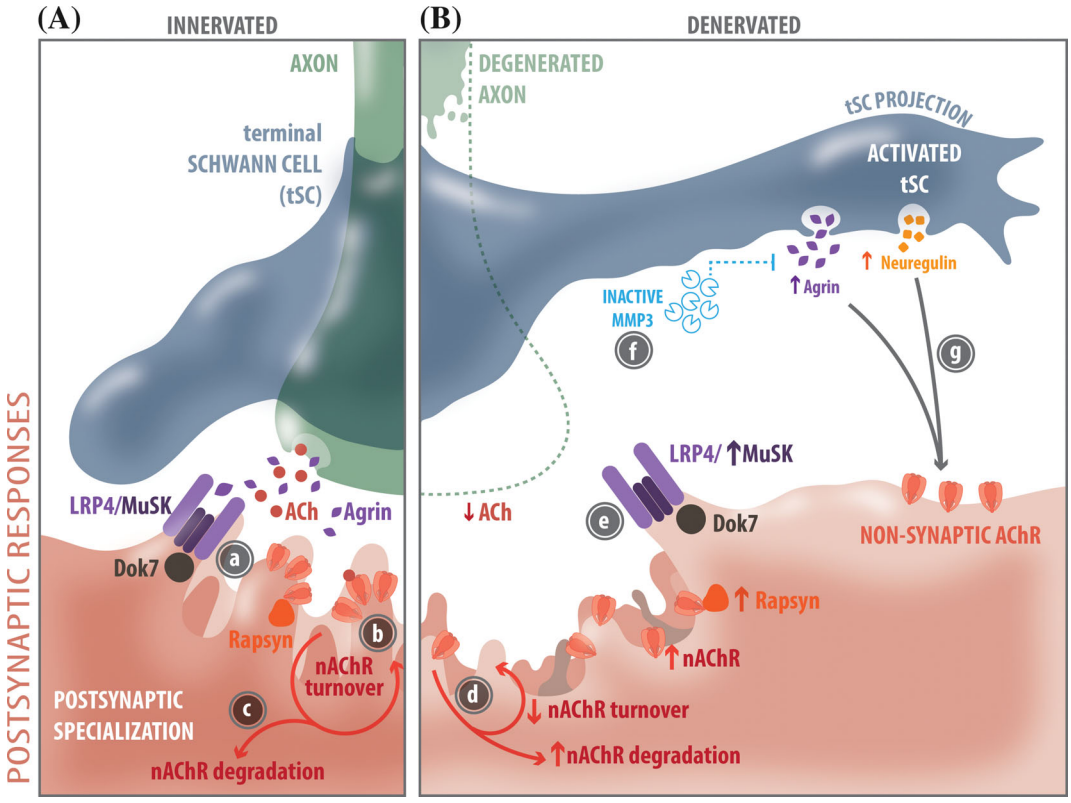

Fig 2. Crucial cells and molecules involved in postsynaptic responses in the regenerative niche at the vertebrate neuromuscular junction (NMJ). In normally innervated NMJs (A), agrin/LRP4/MuSK signalling is triggered by nerve terminal-secreted agrin, which leads to Dok7 activation with the subsequent clustering of nAChRs mediated by rapsyn, specifically in regions that correlate with presynaptic active zones (a). Importantly, nAChR surface levels are stable and are maintained by an active turnover process (b). Distinct routes modulate either positively or negatively nAChR levels in the postsynaptic membrane, including nAChR recycling, *de novo* synthesis, or nAChR degradation (c). Skeletal muscle fibre denervation (B) leads to the loss of muscle membrane organization (d), along with decreased nAChR stability at the cell surface, triggering an increase in nAChR degradation over nAChR turnover, thus decreasing nAChR levels after long-term denervation. Moreover, in order to maintain NMJ organization, effectors of the agrin/LRP4/MuSK pathway increase their expression after NMJ injury (e), whereas the matrix metalloproteinase MMP3 secreted by terminal Schwann cells becomes inactive (f) avoiding agrin processing and therefore increasing agrin levels in the synaptic region. Also, terminal Schwann cells secrete agrin and neuregulin specifically at their extended processes (g), leading to nAChR synthesis in non-synaptic muscle regions. ACh, acetylcholine; AChR, acetylcholine receptor; Dok7, downstream of tyrosine kinase 7; LRP4, lipoprotein receptor related protein-4; MMP3, matrix metalloproteinase 3; MuSK, muscle-specific tyrosine kinase receptor; nAChR, nicotinic acetylcholine receptor; rapsyn, 43 kDa receptor-associated protein of the synapse.

developmental stages onwards. Despite the well-described function of myelinating Schwann cells in the propagation of presynaptic action potentials, the roles that terminal Schwann cells play in NMJ formation, maintenance, and remodelling, as well as in synaptic function and plasticity, are still emerging (Ko & Robitaille, 2015; Alvarez-Suarez, Gawor & Proszynski, 2020).

(2) Establishment of mature NMJs

Throughout early postnatal development, the NMJ achieves its mature complex shape due to drastic modifications in both pre and postsynaptic sites. In murine models, initial postsynaptic densities are poly-innervated by up to 12 presynaptic motor axon branches (Tapia *et al.*, 2012). During the first two weeks of postnatal life, a 'synaptic elimination' step takes place. At this stage, an activity-dependent competition process between axon terminals results in the retraction of all except one motor axon, giving rise to a single synapse per myofibre (Redfern, 1970; Favero, Busetto & Cangiano, 2012; Turney & Lichtman, 2012; Smith *et al.*, 2013). Molecular clues modulating this pruning event include ligands secreted by motor axons, terminal Schwann cells, and muscle fibres that bind and activate diverse presynaptic receptors, such as muscarinic AChRs, adenosine receptors, and the tropomyosin-related kinase B neutrotrophin receptor, leading to the activation of signalling pathways that ultimately reduce the number of innervating axons (Tomas *et al.*, 2017; Lee, 2020). Terminal Schwann cells play a crucial role in this process by decoding synaptic activity signals at competing nerve terminals of poly-innervated NMJs, inducing the retraction of 'weak' motor axons (Darabid, Arbour & Robitaille, 2013).

Specifically, terminal Schwann cells promote the turnover of synaptic motor axon contacts by phagocytosis and by direct competition for the postsynaptic domain (Smith *et al.*, 2013; Santosa *et al.*, 2018). Remarkably, the mechanisms described at the NMJ have been crucial to understanding synaptic elimination in central synapses, a key sculpting process to refine neuronal connectivity and improve synaptic specificity (Chung & Barres, 2009; Nadal *et al.*, 2016).

The postsynaptic domain is also strongly modified throughout NMJ maturation. During this process, AChR subunit composition varies from an embryonic $\alpha_2\beta\gamma\delta$ to a mature $\alpha_2\beta\varepsilon\delta$ conformation. The exchange of γ- for ε-subunits modifies several features of the assembled AChR pentamers, such as their stability (see Section IV), as well as their kinetic and transmission properties (Mishina *et al.*, 1986; Gu & Hall, 1988; Villarroel & Sakmann, 1996). Morphologically, the initial small 'plaque'-like uniform AChR densities are transformed into larger aggregates with complex branches in a 'pretzel'-like shape (Steinbach, 1981; Slater, 1982; Marques *et al.*, 2000; Lanuza *et al.*, 2002; Bolliger *et al.*, 2010), concomitant to the invagination of the sarcolemma into primary and secondary folds (Marques *et al.*, 2000). These folded structures concentrate both: (*i*) AChRs at the edges, in direct apposition to the presynaptic active zones (the axonal domains containing synaptic vesicle clusters and membrane proteins that allow for efficient neurotransmitter release), and (*ii*) the voltage-gated sodium channel Nav1.4 in their depth, which favours action potential generation for subsequent muscle contraction (Slater, 2008*a*,*b*; Wu *et al.*, 2010; York & Zheng, 2017). Despite detailed morphological characterization (Marques *et al.*, 2000), little is known regarding the molecules controlling NMJ maturation. As postsynaptic maturation implies the formation of non-innervated microdomains of the sarcolemma devoid of AChR clusters within the initial postsynaptic plaques, 'anti-clustering' signals might be required for NMJ maturation. *In vitro* experiments have revealed that podosomes, complex molecular structures comprised of filamentous actin and actin-regulating proteins, could be crucial to consolidate the plaque-to-pretzel transition, as their presence has been spatiotemporally related to the topological changes occurring in AChR aggregates (Proszynski *et al.*, 2009; Pezinski *et al.*, 2020). Recent findings show that the scaffolding intracellular protein microtubule-actin crosslinking factor 1 (MACF1) is a key regulator of podosome formation *in vitro*. MACF1 interacts with the AChR-binding protein rapsyn and serves as a postsynaptic organizer of microtubule and actin-associated proteins *in vivo* (Oury *et al.*, 2019). Moreover, the induction of podosome-like structures by extracellular matrix proteins has also been related to the assembly and topological remodelling of AChR clusters (Chan *et al.*, 2020). Mechanistically, this event is mediated by the trafficking and insertion of membrane-type 1 matrix metalloproteinase (MT1-MMP) to the cell surface which, in turn, is mediated by cytoskeleton-dependent mechanisms (Chan *et al.*, 2020). Furthermore, distinct molecules derived either from the NMJ pre-, peri-, or postsynaptic components have been described to play specific roles in refining the structural, molecular and functional organization of the NMJ throughout its maturation process (Shi, Fu & Ip, 2012). For instance, laminins, main components of the basal lamina at the synaptic cleft, act at both sides of the neuromuscular synapse to help the clustering of Cav2.2 calcium channels and organize active zones in presynaptic terminals, but also to promote the maturation of the postsynaptic muscle membrane (Kummer *et al.*, 2004; Fox *et al.*, 2007; Nishimune *et al.*, 2008; Dombert *et al.*, 2017). Intracellularly, the Ras-homologous-guanine nucleotide exchange factor (Rho-GEF) ephexin-1 has been shown to play an indispensable role in NMJ maturation by regulating the stability of AChR aggregates through RhoA (Shi *et al.*, 2010). Additionally, several auxiliary proteins belonging to the dystrophin glycoprotein complex (DGC), including dystrophin, α- or β-dystroglicans, α-synthrophin, and α-dystrobrevin-1 participate in NMJ maturation both *in vitro* and *in vivo* (reviewed in Belhasan & Akaaboune, 2020). More recently, the two-pass transmembrane protein vezatin was identified by an unbiased approach as an AChR-associated protein. Functionally, vezatin plays a crucial and specific role in the structural elaboration and stabilization of AChR aggregates towards a mature pretzel-like shape (Koppel *et al.*, 2019).

A drastic modification of NMJ structure also occurs in aged NMJs. In aged rodents (12 months old onwards), NMJs display an increased area of postsynaptic endplates in which AChR aggregates re-distribute in small fragments with impaired pre- and postsynaptic overlapping (Balice-Gordon, 1997). In mice, fragmented NMJs are also a primary feature of detrimental conditions that very often lead to muscle denervation. NMJ fragmentation associated with muscle denervation is observed after nerve injury or muscle fibre damage, as well as in murine models of muscular pathologies (e.g. muscular dystrophies) and neurodegenerative motor diseases, such as amyotrophic lateral sclerosis, Charcot-Marie-Tooth disease, and spinal muscular atrophy with respiratory distress, characterized by motor neuron death and consequent NMJ denervation (Bogdanik *et al.*, 2013; Rudolf *et al.*, 2014; Pratt *et al.*, 2015; van der Pijl *et al.*, 2016; Villalon *et al.*, 2018). Strikingly, a fragmented distribution of AChR clusters is normally present in healthy mature NMJs of other species, such as lower vertebrates and some mammals, including humans. In this regard, it should be noted that human coin-shaped NMJs are smaller, less complex, and exhibit thinner nerve terminals than murine ones (Jones *et al.*, 2017). Also, human NMJs are morphologically stable across the adult lifespan and do not exhibit signs of age-related degeneration (Jones *et al.*, 2017). Even though NMJ fragmentation traditionally has been considered a sign of synaptic weakness, this concept has been discussed in depth and questioned in a recent review (Slater, 2020). By integrating morphological and functional evidence the author suggests that rather than being a sign of synaptic weakness, NMJ fragmentation might be interpreted as an outcome of synaptic adaptation after injury (Slater, 2020).

II. NMJ DYSFUNCTIONS AND REGENERATION

Peripheral nerve injury can be mimicked in animal models following protocols of either nerve crush (axonotmesis), in which axons are disrupted but the connective tissue sheaths and basal lamina remain intact, leading to effective axonal regeneration and function restoration, or nerve cut (neurotmesis), in which axons, connective sheaths, and basal lamina are interrupted, leading to null functional recovery (Savastano *et al.*, 2014). Delayed reinnervation can be experimentally accomplished by sequential nerve crushes, as well as by complete nerve transection followed by re-suture at different time points (neurorraphy) (Ma *et al.*, 2011; Sakuma *et al.*, 2016). Cumulative evidence using these experimental paradigms has revealed that NMJ regeneration may be achieved due to a balance amongst structural stability, plasticity, and repair, together with synaptic efficacy (Ko & Robitaille, 2015). However, the remarkable ability of the peripheral nervous system to regenerate only occurs through the establishment of permissive environments for reinnervation. These environmental conditions work together to allow key steps for successful regeneration, including: (*i*) the Wallerian degeneration of the axonal stump distal to the injury site, a process characterized by the disintegration of the axonal cytoskeleton and membrane rupture, both required for subsequent axonal regeneration. Axonal degeneration is followed by the degradation of the myelin sheath and the recruitment of macrophages, which serve to promote vascularization and the clearance of degeneration debris (Rotshenker, 2011; Klimaschewski, Hausott & Angelov, 2013). Importantly, at the injury site, as axons become disrupted calcium ions pass through the axolemma, triggering a signalling cascade that leads to membrane repair and to the formation of a growth cone. Also, vesicles transported throughout the axon are assembled at the injury site forming a cap that seals the disrupted axonal membrane (Hill, Coleman & Menon, 2016). (*ii*) The de-differentiation ('activation') of myelinating Schwann cells towards a proliferative phenotype, which originates tube-like endoneurial tracks inside each of the basal lamina tubes, the bands of Büngner, that will enclose regenerating axons. Here, Schwann cells provide essential substrates and guidance cues for regenerating axons travelling towards their target muscle fibres (Jessen & Mirsky, 2005; Scheib & Hoke, 2013). (*iii*) The stabilization of denervated muscle postsynaptic domains (see Section IV).

Analyses of the hind-limb tibialis anterior muscle in mice after sciatic nerve crush show Wallerian degeneration accompanied by complete denervation of motor endplates one week after injury. Partial reinnervation observed after two weeks turns into poly-innervated NMJs after 3–4 weeks. Restoration of a 1:1 motor axon to muscle fibre ratio – comparable to uninjured control NMJs – is reached after six weeks (Magill *et al.*, 2007). This morphological recovery is accompanied by functional motor recovery of the sciatic nerve, as walking track analyses showed a significant improvement three weeks after injury. Pre-injury values were observed six weeks after injury (Magill *et al.*, 2007). In contrast to these dynamic changes observed in the presynaptic apparatus following nerve damage, the gross morphology of the postsynaptic apparatus remains intact within the period between denervation and reinnervation, revealing an intrinsic resilience of the postsynaptic machinery upon denervation (Magill *et al.*, 2007).

Strikingly, although adult mammalian peripheral axons do regenerate, this process does not necessarily result in successful functional recovery in human injuries; indeed, proximal injuries and delayed interventions result in poor functional outcomes (Hoke, 2006). This is at least partially due to changes in the damaged nerve and in the denervated muscle, which make the distal environment increasingly non-permissive for regeneration (Sulaiman & Gordon, 2000). Sakuma *et al.* (2016) elegantly confronted the paradigm that morphological recovery is the exclusive goal to accomplish motor function restoration through NMJ regeneration. In mice and rats subjected to a delayed reinnervation protocol, they verified that regenerating nerve fibres reach motor endplates and reinnervate postsynaptic domains, thus recovering NMJ morphology to a comparable extent to that observed in control NMJs. Furthermore, complete sensory recovery was achieved after prolonged denervation. However, after a critical reinnervation period (>35 days), even though a morphological recovery of NMJ structure was observed by proper pre- and postsynaptic apposition, this rescue effect was not associated with a positive functional outcome in distal muscles, as evidenced by impaired behavioural and electrophysiological performance (Ma *et al.*, 2011; Sakuma *et al.*, 2016). These findings suggest that the absence of motor recovery after traumatic nerve injury may not be due to the lack of muscle fibre reinnervation, nor to a permanent retraction of motor axons, but rather to impaired synaptic functionality.

Based on the aforementioned evidence, we suggest that a regenerative niche at the mature NMJ must ensure two fundamental steps for successful NMJ regeneration: the arrival of incoming regenerating axons to denervated postsynaptic muscle domains for reinnervation, and the maintenance of those postsynaptic domains, in morphological and functional terms. Below, we summarize evidence describing the cells and molecules that could coordinate the assembly of a dynamic NMJ regeneration niche.

III. A REGENERATIVE NICHE AT THE NMJ: HOW DO MOTOR AXONS REACH AND REINNERVATE MUSCLE FIBRES?

Terminal Schwann cells are active effectors of synapse elimination, modulation, and fine-tuning throughout NMJ establishment and maturation (reviewed in Arbour, Vande Velde & Robitaille, 2017; Ko & Robitaille, 2015; Sugiura & Lin, 2011). The role of terminal Schwann cells on NMJ regeneration has been well described at the morphological

level, as they are essential cells to guide regenerating nerve terminals for successful NMJ repair (Reynolds & Woolf, 1992; Son & Thompson, 1995). It was first demonstrated that two weeks after injury-induced NMJ denervation, terminal Schwann cells extend long cellular processes beyond synaptic sites. These cellular processes retract only if reinnervation occurs, suggesting that terminal Schwann cell activation is required for NMJ regeneration (Reynolds & Woolf, 1992) (Fig. 1B). Indeed, Son & Thompson (1995) demonstrated that regenerating motor axons are guided to denervated NMJs following terminal Schwann cell processes. Using a partial denervation paradigm in the soleus muscle (in which innervated NMJs were in close proximity to denervated ones) they demonstrated that terminal Schwann cell projections from denervated NMJs form bridges with adjacent innervated NMJs. As a result, axons from innervated NMJs project 'escaped fibres' that grow along terminal Schwann cell bridges to reinnervate the NMJ (Fig. 1B) (Son & Thompson, 1995). Most of the stability of terminal Schwann cell bridges between denervated and innervated endplates relies on NMJ activity. In fact, administration of either pre- or postsynaptic toxins (that block synaptic transmission) immediately after partial denervation strongly decreased the number of terminal Schwann cell bridges (Love & Thompson, 1999). Moreover, recent *in vivo* evidence has shown that altered patterns of reinnervation observed after severe nerve injury can be explained by an altered guidance of motor axons through terminal Schwann cell processes towards a muscle different than the one they previously occupied (Kang, Tian & Thompson, 2019).

Although this evidence demonstrates that terminal Schwann cell sprouts serve as guidance substrates for axonal escaped fibres after nerve injury, the molecular mechanisms involved in terminal Schwann cell activation have not been fully elucidated. In this context, different terminal Schwann cell-derived proteins alter their gene expression after denervation, such as the growth-associated protein-43 (GAP-43), the low-affinity nerve growth factor receptor $p75^{NTR}$, the intermediate filament protein nestin, the cell adhesion molecule CD44, and the transcriptional factor zinc-finger proliferation protein 1 (reviewed in Sugiura & Lin, 2011). Interestingly, terminal Schwann cells from denervated frog NMJs up-regulate the expression of the glial fibrillary acidic protein (GFAP) (Georgiou *et al.*, 1994), a similar response to that of astrocytes following injury of central nervous system synapses (Stafford *et al.*, 1990). Of note, GFAP expression was reduced upon electrical stimulation of the distal nerve stump (i.e. the part still attached to the muscle), suggesting that presynaptic inputs regulate GFAP expression in terminal Schwann cells (Georgiou *et al.*, 1994). Thus, one good candidate presynaptic regulator of GFAP expression is the neurotransmitter acetylcholine, as it binds to nicotinic AChRs in the muscle membrane but also to muscarinic AChRs in Schwann cells (Georgiou, Robitaille & Charlton, 1999) (b in Fig. 1A). Experiments using the agonist muscarine in denervated frog muscles prevented GFAP up-regulation in terminal Schwann cells (Georgiou *et al.*, 1999), suggesting that impaired signalling through muscarinic AChRs in terminal Schwann cells is involved in their activation. In addition, *in vivo* analyses showed that the muscarinic AChR antagonist atropine induced the formation of axonal processes escaping from synaptic regions in a similar pattern to that observed after NMJ denervation; indeed, atropine-induced axonal escaped fibres followed GFAP-expressing terminal Schwann cell processes (Wright *et al.*, 2009) (c in Fig. 1B). Thus, the blockade of synaptic activity-dependent release of acetylcholine due to NMJ denervation not only precludes muscle membrane depolarization through nicotinic AChRs, but also acts as an activation switch in terminal Schwann cells through muscarinic AChRs to help motor axon guidance for NMJ regeneration (Sugiura & Lin, 2011; Ko & Robitaille, 2015; Arbour *et al.*, 2017).

Denervation elicits major cellular and molecular alterations, as it not only deprives the NMJ microenvironment of motor axons and Schwann cells, but also of retrograde and anterograde signals that normally stabilize either the NMJ presynaptic (e.g. muscle-derived neurotrophic factors) and postsynaptic domains, respectively. One such factor is the Neuregulin family of alternatively spliced factors secreted by presynaptic motor terminals and terminal Schwann cells, which exist as secreted or membrane-bound forms. Neuregulins signal through the ErbB family of tyrosine kinase receptors activating signalling pathways involved in multiple cell responses, including the formation, differentiation, and survival of Schwann cells (Darabid, Perez-Gonzalez & Robitaille, 2014). Genetic studies targeting Neuregulin-1 as well as its receptors ErbB2 and ErbB3 provide information regarding the role of Schwann cells on NMJ formation and maintenance. While Neuregulin–ErbB signalling impairment leads to terminal Schwann cell ablation (Lin *et al.*, 2000), analyses in Neuregulin-1-, ErbB2- or ErbB3-mutant mice showed that early NMJ assembly was not affected; however, short-term retractions of nerve terminals are observed later in these animals, suggesting an important role of terminal Schwann cells on NMJ maintenance (Riethmacher *et al.*, 1997). Different findings reveal that Neuregulin/ErbB pathways could be also relevant to inducing terminal Schwann cell activation and sprouting after nerve injury (d in Fig. 1B). First, denervation induces the up-regulation of Neuregulin/ErbB pathway effectors in terminal Schwann cells after denervation (Nicolino *et al.*, 2009). Second, functional studies suggest that conditional induction of a constitutively active form of the ErbB2 receptor in Schwann cells of transgenic mice, or Neuregulin-1–III overexpression specifically in motor neurons, is sufficient to alter normal NMJ organization (Hayworth *et al.*, 2006; Lee *et al.*, 2016). Third, overexpression of Neuregulin-1 in motor neurons induces the extension of terminal Schwann cell processes, soma migration, and proliferation, as well as sprouting of the motor nerve terminal along cell processes, mimicking a denervation/reinnervation response (Hayworth *et al.*, 2006). Thus, Neuregulin–ErbB pathways are also involved in terminal Schwann cell responses after injury likely *via* paracrine and/or axo-glial signalling.

Before the damaged nerve terminal leaves the NMJ synaptic region, an early response from terminal Schwann cells is

achieved, suggesting that the degenerating nerve terminal is another source of glial activation. Toxin-dependent presynaptic NMJ degeneration resulted in increased levels of phosphorylated extracellular signal-regulated kinase (ERK) specifically in terminal Schwann cells; indeed, up-regulation of the mitogen-activated protein kinase (MAPK)/ERK signalling pathway was shown to be essential for terminal Schwann cell activation and for NMJ regeneration (Duregotti *et al.*, 2015). Interestingly, presynaptic injury leads to the release of intracellular mitochondria-derived damage-associated molecular patterns (DAMPs) or alarmins (Krysko *et al.*, 2011) (e in Fig. 1B), of which at least mitochondrial DNA and cytochrome C induce ERK phosphorylation in Schwann cell cultures (Duregotti *et al.*, 2015). In addition, the MAPK pathway plays a central role in the control of Schwann cell plasticity and peripheral nerve regeneration *via* ERK1/2 and Jun N-terminal kinase (JNK) *via* c-Jun, a key component of this signalling cascade (Arthur-Farraj *et al.*, 2012).

Once terminal Schwann cell projections reach innervated NMJs (in the partial denervation paradigm) or incoming regenerating motor axons (in the nerve crush injury model), they guide axonal projections to the denervated postsynaptic apparatus. Recent transcriptome-based analyses showed that after motor axon damage, terminal Schwann cells increase the expression and secretion of C-X-C motif chemokine 12α/stromal cell-derived factor 1 (CXCL12α/SDF-1) (Negro *et al.*, 2017), a chemokine involved in a variety of cellular and tissue responses, including early development, organ formation, and a broad variety of responses in the immune and nervous systems (Lu, Grove & Miller, 2002; Lieberam *et al.*, 2005; Zhu *et al.*, 2009). CXCL12α/SDF-1 acts through the C-X-C chemokine receptor type 4 (CXCR4), which is expressed in motor axon terminals, promoting axonal elongation in primary spinal motor neurons (Negro *et al.*, 2017). CXCL12α or CXCR4 gain- and loss-of-function experiments *in vivo* showed accelerated and delayed functional NMJ recovery, respectively, indicating that nerve injury-dependent activation of the CXCL12α–CXCR4 axis is crucial to guiding motor axons to allow for efficient NMJ regeneration (Negro *et al.*, 2017) (f in Fig. 1B). Remarkably, it has been demonstrated recently that the CXCR4 receptor agonist NUCC-390 strongly promotes nerve regeneration and functional NMJ recovery after toxin-dependent presynaptic degeneration or mechanical damage (Negro *et al.*, 2019).

From a clinical viewpoint, it is relevant to consider cumulative evidence showing terminal Schwann cells sprouting in different pathological contexts affecting the NMJ (Santosa *et al.*, 2018). For instance, in muscles from human amyotrophic lateral sclerosis patients, most terminal Schwann cells project extensive cytoplasmic processes and a proportion of NMJs are mainly contacted by terminal Schwann cell projections rather than by their cell bodies (Bruneteau *et al.*, 2015). A similarly altered distribution of terminal Schwann cells has also been described in two different animal models of the disease at stages preceding NMJ denervation (Carrasco, Seburn & Pinter, 2016*b*). Consistently, nerve crush injury results in terminal Schwann cell depletion from synaptic regions and, consequently, in impaired NMJ reinnervation in an amyotrophic lateral sclerosis model (Carrasco *et al.*, 2016*a*). Similarly, a murine model of spinal muscular atrophy exhibits NMJ disorganization and abnormally high nerve sprouting, which correlates to an exacerbated proportion of terminal Schwann cell projections. Also, the number of terminal Schwann cells per NMJ is significantly reduced in the spinal muscular atrophy model, which likely relates to the defects observed in NMJ maintenance in this mouse model (Murray *et al.*, 2013). Even though the NMJs of a mouse model of Duchenne muscular dystrophy exhibit terminal Schwann cells with extensive cytoplasmic processes, their ability to form bridges towards adjacent innervated endplates is reduced compared to controls (Personius & Sawyer, 2005).

Taken together, these findings demonstrate that a specific inter- and intracellular molecular interplay amongst degenerating motor axons and terminal Schwann cells is a highly attractive potential target to manipulate the activation of terminal Schwann cells in order to guide incoming motor axons towards denervated skeletal muscle fibres for successful NMJ regeneration (Fig. 1B).

IV. CELLULAR AND MOLECULAR CLUES IN THE MAINTENANCE OF THE NMJ POSTSYNAPTIC APPARATUS

A well-known hallmark of the denervation of mature NMJs is the re-expression of the embryonic AChR γ-subunit (Witzemann *et al.*, 1987; Goldman & Staple, 1989; Tsay & Schmidt, 1989); however, the functional consequences of this molecular change have not been clearly established. Muscle denervation experiments performed at different ages have shown that immature AChR plaques disassemble faster than mature pretzel-like AChRs (Slater, 1982; Moss & Schuetze, 1987), suggesting that AChR pentamers formed with the foetal AChR γ-subunit ($\alpha_2\beta\gamma\delta$) might have lower stability than those assembled with the mature ε-subunit ($\alpha_2\beta\varepsilon\delta$). Even though most AChRs found at mature denervated endplates contain the γ- instead of the ε-subunit, they maintain a comparatively more stable morphology than immature denervated endplates (Slater, 1982; Moss & Schuetze, 1987). In this regard, although mature pretzel-like AChR aggregates maintain their gross shape for several weeks after nerve injury, detailed analyses have demonstrated injury-dependent loss and gain of AChRs in entire pretzel branches within postsynaptic regions, an effect that increases at longer denervation times (Kang *et al.*, 2014). Understanding the metabolic dynamics of AChRs within the postsynaptic muscle domain in normal and denervated conditions is a first crucial step before potential therapeutic interventions can be designed (b–d in Fig. 2). Following *in vivo* radioactive pulse-chase, fluorescence microscopy, and

biochemical approaches, it has been determined that AChRs of innervated adult NMJs are stable in the muscle membrane, comprising half-life times of around 14 days (Xu & Salpeter, 1997; Akaaboune *et al.*, 1999; Strack *et al.*, 2011). In turn, different muscle-derived AChR populations coexist after denervation (Shyng & Salpeter, 1989; Fumagalli *et al.*, 1990). According to their half-life, they can be classified as slow (13–14 days), medium (5–8 days), and fast (1 day) AChRs (Strack *et al.*, 2011; Strack *et al.*, 2015). The rate at which AChRs are removed from the sarcolemma and subsequently internalized into muscle fibres is increased after denervation, thus leading to AChR instability at the muscle surface (d in Fig. 2B). Importantly, impaired AChR stability is reversed in muscles chronically stimulated after denervation (Levitt & Salpeter, 1981; Andreose *et al.*, 1993; Akaaboune *et al.*, 1999), suggesting that once reinnervated, neurotransmission through the NMJ helps postsynaptic stability and functional recovery. In this regard, the stability of AChRs at the plasma membrane is significantly variable after different stimuli including activity blockade, embryonic development, denervation, ageing, agonist application, muscle atrophy, as well as in pathological conditions such as myasthenia gravis (Burden, 1977; Drachman *et al.*, 1980; Akaaboune *et al.*, 1999; Valdez *et al.*, 2010; Strack *et al.*, 2011). How are the traffic routes followed by internalized AChRs driven towards degradation or recycling? In heterologous CHO-K1/A5 cells stably expressing the adult muscle-type AChRs, internalization occurs *via* a mechanism that depends on Rac (also known as Ras-related C3 botulinum toxin substrate 1), but is independent of clathrin, caveolin and dynamin (Kumari *et al.*, 2008). Once endocytosed, AChRs traffic *en route* through early/late endosomes and are then degraded by a lysosome-dependent mechanism (Kumari *et al.*, 2008). At the *in vivo* NMJ, AChRs are internalized and degraded under basal conditions, as systemic treatment with chloroquine, a lysosomotropic agent that blocks vesicular acidification at the endosomal (Yuyama, Yamamoto & Yanagisawa, 2006) or autolysosomal (Cain & Murphy, 1986) level, leads to the accumulation of AChR endocytic carriers. Notably, the formation of internalized AChR-containing vesicles is stimulated eightfold after denervation, a feature that further increases in chloroquine-treated denervated mice (Wild *et al.*, 2016). Moreover, microscopy and biochemical analyses have shown that chloroquine treatment leads to increased co-localization of AChR-containing vesicles with the standard endocytic regulator RAB5 (Ras-associated binding 5), but not with the autophagy marker microtubule-associated protein 1 light chain 3B (MAP1LC3B) (Wild *et al.*, 2016). These findings suggest that manipulations favouring endocytic rather than autophagic fluxes could control AChR stability within the denervated postsynaptic domain (b–d in Fig. 2).

A second key level for potential therapeutic intervention is to identify molecules and mechanisms that increase AChR stability in the sarcolemma. In this regard, a common feature of most molecular mechanisms helping tissue regeneration is that they were first characterized for their role in embryonic assembly. Although most of the molecules commanding embryonic postsynaptic assembly at the NMJ strongly decrease their expression in muscles towards adulthood, the expression of key postsynaptic proteins, such as the AChRs, rapsyn (Baldwin *et al.*, 1988), and MuSK (Valenzuela *et al.*, 1995) are strongly up-regulated upon NMJ denervation (e in Fig. 2B). In addition, the identification of molecular defects involved in motor pathologies has revealed that the expression of these molecules at the mature NMJ is crucial for synaptic maintenance. Indeed, the presence of autoantibodies blocking the function of AChRs, but also of those targeting MuSK or LRP4, results in different forms of myasthenia gravis (Drachman *et al.*, 1980; Yan *et al.*, 2018; Huijbers *et al.*, 2019), whereas mutations in the genes encoding agrin, MuSK, LRP4, Dok7, and rapsyn are responsible for different congenital myasthenic syndromes (Brugnoni *et al.*, 2010; Maselli *et al.*, 2010, 2012; Palace, 2012; Nicole *et al.*, 2014; Vanhaesebrouck & Beeson, 2019; Rodriguez Cruz *et al.*, 2020). These observations are consistent with experimental deletion approaches in mice. For instance, conditional silencing of the muscle-specific *lrp4* gene specifically at postnatal stages leads to the dismantling of pretzel-like AChR aggregates (Barik *et al.*, 2014). These morphological defects correlate with impaired apposition between pre- and postsynaptic apparatuses and reduced muscle excitability (Barik *et al.*, 2014). LRP4 was also shown to be an essential stop signal for nerve terminals, indicating an additional role in the differentiation of the presynaptic domain (Wu *et al.*, 2012). Together, this evidence reveals that muscle LRP4 is essential for the maintenance of the structural and functional integrity of mature NMJs by playing key roles in both pre and postsynaptic domains. Similarly, *in vivo* electroporation of adult muscles using a short hairpin RNA (shRNA) to silence Dok7 led to impaired motor performance and marked alterations in NMJ morphology (Eguchi *et al.*, 2016). Although AChR clusters were accurately covered by nerve terminals, the size of the NMJ pre and postsynaptic domains was significantly reduced upon Dok7 silencing (Eguchi *et al.*, 2016). Furthermore, Dok7 down-regulation reduced MuSK and AChR expression, suggesting that Dok7 suppression represses MuSK-mediated signalling in the muscle fibre for proper maintenance of the mature NMJ (Eguchi *et al.*, 2016). On the other hand, even though the overexpression of a construct coding for rapsyn coupled to green fluorescent protein (rapsyn-GFP) in adult mice did not affect AChR density in both control and denervated NMJs (Bruneau & Akaaboune, 2010), shRNA-mediated rapsyn silencing resulted in reduced AChR clustering (Kong, Barzaghi & Ruegg, 2004; Martinez-Martinez *et al.*, 2009). Interestingly, the number of secondary folds as well as the protein levels of sodium channels were augmented after rapsyn silencing, which correlated with mild defects in neuromuscular transmission (Martinez-Martinez *et al.*, 2009). In addition, the Yes-Associated Protein (YAP), a major effector of the Hippo pathway, has also been shown to play crucial roles in NMJ formation and functional regeneration (Zhao *et al.*, 2017). The aforementioned evidence reveals the

potential use of muscle-derived proteins controlling NMJ formation as therapeutic targets to help NMJ regeneration.

A noteworthy advance towards the functional targeting of NMJ postsynaptic proteins has been achieved by studies on MuSK. The first hint that MuSK could play an important role in NMJ regeneration came from nerve injury experiments. After sciatic nerve transection, MuSK expression in hind-limb muscles was strongly up-regulated 2–7 weeks after injury (e in Fig. 2B); however, this initial MuSK up-regulation returned to basal levels four weeks after sciatic nerve crush, paralleling muscle reinnervation time (Valenzuela *et al.*, 1995). In these experiments, MuSK expression increased in extrasynaptic regions whereas decreased MuSK levels were detected in synaptic regions (Valenzuela *et al.*, 1995; Bowen *et al.*, 1998), in agreement with evidence showing that local agrin-induced MuSK activation in the muscle subsynaptic region leads to the up-regulation of MuSK and AChR ϵ-subunit expression (Lacazette *et al.*, 2003). Remarkably, recent evidence obtained from a patient carrying a severe early-onset congenital myasthenic syndrome associated with two missense mutations in the MuSK gene has revealed that fine control of MuSK phosphorylation, which commands AChR clustering, is crucial for NMJ structure (Rodriguez Cruz *et al.*, 2020). The concept that MuSK activation could help NMJ maintenance was challenged in a mouse model of amyotrophic lateral sclerosis (Paez-Colasante *et al.*, 2015). Remarkably, NMJ denervation is observed prior to the onset of amyotrophic lateral sclerosis symptoms and before any significant motor axon or cell body loss (Fischer *et al.*, 2004; Pun *et al.*, 2006; Vinsant *et al.*, 2013; Clark *et al.*, 2016; Tallon *et al.*, 2016). A 'dying-back hypothesis' has been proposed, by which early NMJ denervation is the primary step leading to motor neuron death (Moloney, Winter & Verhaagen, 2014). Mice carrying mutated forms of human superoxide dismutase 1, specifically its G93A mutation (hSOD1-G93A), have been widely used as reliable models of amyotrophic lateral sclerosis (Paez-Colasante *et al.*, 2015). In a first genetic approach, hSOD1-G93A mice were crossed with mice expressing threefold more MuSK in muscles than wild-type mice (Perez-Garcia & Burden, 2012), which were previously demonstrated to extend NMJ maintenance in agrin-null mice (Kim & Burden, 2008). In these studies, while denervation was found to reach around 50% at postnatal day 140 (P140) in hSOD1-G93A mice, in double mutants overexpressing MuSK in the hSOD1-G93A background, NMJ denervation remained as low as <10% by P150. In addition, evident muscle denervation began 10 days later in double-mutant mice compared to hSOD1-G93A mice (Perez-Garcia & Burden, 2012), revealing that MuSK activation could play a protective role by helping NMJ stability and therefore preventing the extent of denervation. Remarkably, a pharmacological approach gave rise to comparable positive outcomes in hSOD1-G93A mice, even when NMJ denervation had already started (Cantor *et al.*, 2018). For these experiments, a humanized anti-MuSK antibody that had been demonstrated previously to activate MuSK *in vitro* (Xie *et al.*, 1997) was used. A single antibody injection at early symptomatic stage (P90) resulted in significantly delayed denervation at P110 in hSOD1-G93A mice; moreover, chronic antibody delivery resulted in improved motor performance and decreased motor neuron loss in amyotrophic lateral sclerosis model mice (Cantor *et al.*, 2018). Remarkably, recent studies have shown that the overexpression of Dok7 (Miyoshi *et al.*, 2017) or Neuregulin-1 (Modol-Caballero *et al.*, 2020) by an adeno-associated virus approach in skeletal muscles at pre-symptomatic stages of hSOD1-G93A mice has important protective effects, including delayed NMJ denervation, improved motor function and, consequently, muscle fibre atrophy suppression accompanied by improved coordination and motor performance (Miyoshi *et al.*, 2017; Modol-Caballero *et al.*, 2020).

The evidence targeting MuSK, Dok7 or Neuregulin-1 expression demonstrates that therapeutic manipulation of muscle proteins helping postsynaptic assembly and maintenance constitutes a very interesting therapeutic alternative to prevent denervation or to extend postsynaptic resilience to allow for optimal NMJ functional reinnervation (a and e in Fig. 2).

It is interesting to note that active terminal Schwann cells are also able to secrete ligands that regulate postsynaptic proteins involved in NMJ organization and maintenance (f and g in Fig. 2B). Indeed, terminal Schwann cells at the frog NMJ express the B8-active isoform of agrin (Yang *et al.*, 2001), whose levels increase during NMJ regeneration two weeks after nerve transection. In addition, genetic deletion of terminal Schwann cell-derived MMP3, a matrix metalloproteinase that cleaves synaptic basal lamina molecules, including agrin (VanSaun *et al.*, 2007), stabilizes the motor endplate after injury, leading to improved functional recovery (Chao *et al.*, 2013). Together, these findings suggest that terminal Schwann cells provide the NMJ microenvironment of active agrin after nerve injury (f and g in Fig. 2B). In uninjured NMJs, terminal Schwann cells also secrete Neuregulin-2 (encoded by a *neuregulin-1*-related gene) that activates *in vitro* AChR transcription in muscle cells expressing the ErbB4 receptor (Rimer *et al.*, 2004) (g in Fig. 2B). Interestingly, the distribution of ectopic AChR clusters observed after NMJ denervation matched that of active terminal Schwann cell projections (Yang *et al.*, 2001), supporting the idea that these cells collaborate and can be targeted to maintain the NMJ postsynaptic domain after nerve injury.

V. A FOURTH (RATHER UNEXPECTED) CELLULAR PARTNER AT THE NMJ REGENERATIVE NICHE: MUSCLE SATELLITE CELLS

Recent evidence suggests that another cell type is required for efficient NMJ regeneration. Muscle satellite cells are muscle stem cells that normally reside in a quiescent state all along the muscle fibre between the myofibre and its basal

lamina (Mauro, 1961). In response to muscle injury, satellite cells divide to provide myogenic precursors that will form new muscle fibres or fuse to injured ones, accounting for skeletal muscle regeneration (Yin, Price & Rudnicki, 2013). Mice engineered for conditional depletion of satellite cells displayed NMJ disruption, muscle atrophy, and force decline. In addition, satellite cell depletion resulted in deficient reinnervation and impaired postsynaptic morphology at regenerating NMJs after sciatic nerve injury. Interestingly, satellite cells in close proximity to regenerating NMJs become preferentially activated and fuse to the muscle fibres after sciatic nerve injury, suggesting that neuromuscular disruption leads to regional satellite cell activation (Liu *et al.*, 2015). It was subsequently shown that satellite cells are the source of subsynaptic nuclei, participating in the maintenance of adult NMJs. Correspondingly, the decrease in the satellite cell population is directly related to the decrease in subsynaptic myonuclei observed in degenerating aged NMJs (Liu *et al.*, 2017). Recent single-cell transcriptomic analyses show that different gene-expression programs are triggered in satellite cells after neuromuscular degeneration or muscle trauma, revealing the existence of different subsets of satellite cells for each condition. This notion is reinforced by data revealing that although neurodegeneration activates satellite cells in a comparable fashion to ageing, aged muscles display impaired fusion of satellite cells after NMJ denervation; in turn, genetic rescue of motor neurons in an amyotrophic lateral sclerosis model restores the number of satellite cells to that seen in young healthy muscles (P. Ulintz, J. Larouche, M. Mohiuddin, J.C. Macias, S.J. Kurpiers, W. Liu, J.J. Choi, L.A. Brown, J.F. Markworth, K. de Silva, B.D. Levi, S.D. Merajver, J.V. Chakkalakal, Y.C. Jang, S.V. Brooks & C.A. Aguilar, unpublished data). Even though this cumulative evidence supports the notion that satellite cells are attractive targets to improve NMJ regeneration, the molecular mechanisms involved in their role in NMJ maintenance still remain unknown.

VI. CONCLUSIONS

(1) In models of nerve injury and motor diseases, most therapeutic approaches have focused on improving nerve repair or preventing neurons from dying. Few studies have focused on the initial degeneration of the neuromuscular synapse. Based on the evidence discussed within this review, possible therapeutic interventions should include, on the one hand, motor axon growth and guidance towards the denervated postsynaptic domain and, on the other, maintenance of the synaptic properties of those domains.

(2) Although loss-of-function experiments have provided potential target molecules to intervene in both processes, such as LRP4, Dok7, or MMP3 down-regulation, gain-of-function experiments in injured or degenerating NMJ models are still relatively scarce. However, some intervention studies are emerging. One remarkable example of this in the presynaptic side is the application of the CXCR receptor agonist NUCC-390, or recombinant CXCL12α *in vivo*; both interventions accelerate neurotransmission rescue after toxin- and mechanically induced nerve damage (Negro *et al.*, 2017). At the postsynaptic side, genetic and most importantly, antibody-mediated activation of MuSK prevents NMJ denervation in a model of amyotrophic lateral sclerosis (Cantor *et al.*, 2018). Also, muscle overexpression of Dok7 (Miyoshi *et al.*, 2017) or Neuregulin-1 (Modol-Caballero *et al.*, 2020) through a gene therapy approach has shown important protective responses in animal models of the same disease. From a clinical viewpoint, these studies show that manipulation of NMJ postsynaptic proteins could result in a significant improvement in the quality of life of patients with amyotrophic lateral sclerosis.

(3) An additional important goal of possible therapeutic interventions is to maximize the chances that local positive effects on NMJ regeneration will not be accompanied by undesired side effects in other body regions. In this regard, a recent article showed that local injection of vascular endothelial growth factor (VEGF) plus insulin-like growth factor-1 (IGF-1)-containing hydrogels, specifically at the distal site of sciatic nerve injury (i.e. towards the NMJ), promoted functional reinnervation and muscle regeneration (Raimondo *et al.*, 2019). This study also reveals that NMJ regeneration strategies are not only useful after damage or degeneration, but also for muscle transplantation interventions, such as those focused on facial nerve rescue after paralysis, where successful functional NMJ regeneration must also take place.

(4) In summary, defining the cellular and molecular components of the regenerative NMJ niche, together with effective time frames and intervention methods where the NMJ is compromised, should concentrate future research efforts to achieve successful functional recovery of neuromuscular connectivity.

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